Carbohydrate Research, 149 (1986) 225–239
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

D-GALACTOSYLTRANSFERASE AND ITS ENDOGENOUS SUBSTRATES IN CHICK EMBRYO FIBROBLASTS TRANSFORMED BY ROUS SARCOMA VIRUS*

DANIEL K. PODOLSKY, DEBORAH A. FOURNIER, AND KURT J. ISSELBACHER
Gastrointestinal Research Laboratories, Massachusetts General Hospital and Harvard Medical School, Boston, 02114, Massachusetts (U.S.A.)
(Received April 8th, 1985; accepted for publication in revised form, November 7th, 1985)

ABSTRACT

UDP-D-galactose: 2-acetamido-2-deoxy-β-D-glucopyranosyl 4-β-D-galactosyltransferase (GalTase) activity was purified, from primary chick embryo fibroblast (CEF) transformed by a temperature-sensitive, Rous sarcoma virus mutant (CEF-RSV), by chromatography on an affinity resin prepared with monoclonal antibodies to GalTase. Cellular glycopeptides from CEF, as well as CEF-RSV, maintained at permissive (37°) [CEF-RSV (37°)] and nonpermissive temperatures (41°) [CEF-RSV (41°)], were solubilized and galactosylated *in vitro* by incubation with purified GalTase substrates, composed of at least six discrete complex glycopeptides having bi- to tetra-antennary structures. The glycopeptides isolated from transformed cells, CEF-RSV (37°), included the six types observed in nontransformed cells, but demonstrated alterations in their relative amounts, including an increase in the content of a glycopeptide containing 3 mannose and 4 glucosamine residues. Furthermore, two additional complex-type glycopeptides were isolated from CEF- but demonstrated alterations in their relative amounts, including an increase in the content of a glycopeptide containing 3 mannose and 4 glucosamine residues. Furthermore, two additional complex type glycopeptides were isolated from CEF-RSV (37°). These malignant transformation-related glycopeptides were partially characterized and found to represent tri- and tetra-antennary complex glycopeptides. Endogenous galactosylation appeared to have occurred in a branched, nonspecific manner in these transformed cell-derived glycopeptides. These findings indicate that transformed cells may contain a greater preponderance of more highly branched, complex oligosaccharides which are randomly galactosylated at non-reducing termini by cellular GalTase.

*This work was supported by a grant (CA-31277) from the National Cancer Institute, National Institutes of Health.

0008-6215/86/$ 03.50

INTRODUCTION

Although the role of glycoconjugates in the maintenance of cellular integrity, and regulation of growth and differentiation remains uncertain, attention has focused on the potential importance of glycoprotein structures and biosynthesis in the altered behavior resulting from malignant transformation. It has been suggested that cell surface glycoproteins participate in properties characteristically altered during malignant transformation, including cell adhesion, substrate dependence, morphology, and primary and metastatic tumorigenicity. Several laboratories have examined transformation-related changes in the oligosaccharide side-chains of cellular glycoproteins or glycosyltransferase activities that are responsible for the production of these substances. Although studies comparing glycoproteins of transformed and nontransformed counterparts have failed to demonstrate alterations unique to transformed cells, collectively they indicate the association of the transformed state with a few general alterations in cellular-glycopeptide composition[1–11]. Thus, a large number of reports have noted an increase in higher-molecular-weight, sialylated, complex-type glycopeptides and decrease in lower-molecular-weight, intermediate, and high-mannose glycopeptides[1–5,10,11]. Still others have identified a family of blood group-related oligosaccharides which may be associated with the malignant phenotype[12]. Similarly, many investigators have demonstrated a wide variety of alterations in glycosyltransferase activities in association with viral transformation. These have included both apparent increased and decreased levels of various activities, as well as the emergence of relatively distinctive electrophoretic forms[13]. In this regard, a number of studies have demonstrated alterations in UDP-D-galactose: 2-acetamido-2-deoxy-β-D-glucopyranosyl 4-D-galactosyltransferase (GalTase) activity in conjunction with malignant transformation *in vitro* and cancer *in vivo*. However, past studies have focused on either structural alterations in glycoproteins or enzymic activities, but not on both. Interpretation of the significance in either case is limited; on the one hand, structural alterations reflect changes in ambient glycosyltransferase activities whereas, on the other hand, alterations in cell behavior must be related to glycoprotein products and not to the biosynthetic precursors. Further, it may be difficult to assess whether alterations in cellular glycoconjugates reflect changes in biosynthetic activities or their available substrates. In order to define the relationship of reported alterations in glycoproteins to GalTase activities following malignant transformation, we have both isolated GalTase activity from transformed primary cell cultures and defined its natural substrates in those cells as well as in nontransformed counterparts.

EXPERIMENTAL

Analytical methods. — Descending paper chromatography was performed on Whatman 1MM in 5:5:1:3 (v/v) ethyl acetate–pyridine–acetic acid–water (solvent *A*). High-voltage paper electrophoresis was performed on Whatman 3MM in 0.2M

borate buffer, pH 9.0. Sugar standards were localized on paper with alkaline $AgNO_3$ reagent after treatment with periodate. Determination of radioactivity on paper was accomplished by placing 1-cm section strips in 10 mL of a toluene-based scintillation fluid. T.l.c. was performed on Silica gel G plates in 3:3:2 (v/v) butanol–acetic acid–water (solvent *B*) for 16 h. Oligosaccharides were located with the orcinol spray[14]. Carbohydrate composition was determined by g.l.c. following methanolysis, *N*-reacetylation, and derivatization with a silylating reagent as previously described[15], in a Varian 6000 gas chromatograph equipped with a column (3 m × 3 mm) of 3% SE-30 or OV-17 resin and a Hewlett–Packard 3390A integrator.

Methylation analysis. — Methylation of the native and desialyzed oligosaccharides was performed with iodomethane in the presence of methylsulfinyl carbanion, in dimethyl sulfoxide[16]. The permethylated oligosaccharides recovered from the reaction mixtures by extraction with chloroform were dried and purified by t.l.c.[17]. Following hydrolysis, reduction, and acetylation, the mixtures of partially methylated sugar alcohols were analyzed by gas chromatography[18,19]. The temperature was programmed at 1°/min from 130–210°. The peaks were identified by comparison with migration of standard compounds as previously described[20–22]. Quantitation of each peak was based on response factors determined with standard methylated derivatives, as previously described[18,21], with a standard error of ±16%.

Enzymes. — Beef kidney α-L-fucosidase was obtained from Boehringer–Mannheim Biochemicals; jack bean α- and β-D-mannosidases and *N*-acetyl-β-D-glucosaminidase were purchased from Sigma Chemical Co. (St. Louis, MO). Additional *N*-acetyl-β-D-hexosaminidases, α- and β-D-galactosidases, and α-L-fucosidase were purified from culture fluid of *D. pneumoniae* according to the method of Glasgow *et al.*[23]. One unit of glycosidase activity is defined as that amount of enzyme required to hydrolyze 1 μmol of appropriate *p*-nitrophenyl glycoside substrate per min at 37°.

Cell culture. — Primary CEF cell cultures were prepared from pathogen-free, 10-day chick embryos, as previously described[24], and maintained at 41°. Cultures were infected on day 3 after seeding with a temperature-sensitive, RSV mutant (Ts68) maintained in this laboratory (1 plaque forming unit/cell by plaque focus assay). Noninfected and infected cells were maintained at either 41° (nonpermissive temperature), or transferred to 37° (permissive temperature) 24 h after infection. Secondary cultures were prepared by resuspending the trypsinized cells in roller bottles at a density of 1×10^7 cells/100 mL of medium per bottle.

Enzyme degradation. — Enzymic hydrolysis of oligosaccharides was performed by incubating the substrates at 37° for 24–36 h with α-L-fucosidase, α- and β-D-mannosidase, α- or β-D-galactosidase, or *N*-acetyl-β-D-hexosaminidase in 0.5M sodium citrate buffer, pH 4.0. Following incubation with a single enzyme or a group of enzymes, reaction mixtures were heated in a boiling-water bath for 5 min and an aliquot of the mixture was analyzed for the liberated monosaccharide(s) by paper chromatography or by determination of radioactivity. Residual oligosaccharides

were separated from released monosaccharides by gel filtration on Bio-Gel P-2 column (0.9 × 40 cm) and their composition determined by g.l.c.[15].

Purification of CEF-RSV (37°) GalTase activities. — GalTase was assayed radiochemically as described[25]. It was purified from CEF-RSV (37°) by modification of methods used to purify soluble GalTase from other sources[26,27]. The cells were harvested and washed three times with phosphate-buffered saline, and the crude soluble GalTase was obtained in buffer containing 1% of 3[(3-cholamidopropyl)dimethylammonio] 1-propane sulfonate (CHAPS) (Sigma) as described[26]. Initial purification was accomplished by sequential chromatography on α-lactalbumin–Sepharose 4B and *N*-acetylglucosamine–Sepharose 4B resins as previously described[27]. Final purification was achieved by chromatography on an affinity resin prepared[28] by coupling an anti-GalTase monoclonal antibody (V10) to Sepharose 4B.

Preparation of cellular glycoproteins and [^{3}H]galactosylation by CEF-RSV (37°) GalTase. — Proteins of cells [CEF (37°), CEF (41°), CEF-RSV (38°), and CEF-RSV (41°)] were endogenously labelled for 2 h prior to harvest by addition of L-[^{141}C]leucine (2.9 MBq per roller bottle, sp. act. 12.6 GBq/mmol). When semi-confluent, the cells were harvested with a rubber policeman on ice; in typical experiments, cells from 25–50 roller bottles were pooled. The cells were briefly centrifuged (800*g* for 10 min) and the pellet was washed 3–5 times in phosphate-buffered saline (PBS) (0.154M NaCl–10mM $NaHPO_4$ buffer, pH 7.2). The cell pellet was resuspended in PBS (~10 vol.) and glycoproteins were solubilized by exhaustive sonication with a Branson S-75 sonifier (8 bursts of 15 s at power setting No. 6). The sonicated material was centrifuged at 105 000*g* for 60 min and soluble glycoproteins representing 79–88% of the total radioactivity were obtained as the resulting supernatant. The soluble glycoproteins were dialyzed against water (0–4°) and lyophilized.

Galactosylation was accomplished by incubating the soluble glycoproteins (5.0 mg/mL of 10mM sodium cacodylate, pH 7.4) with 500 μCi UDP-D-[^{3}H]galactose (18.5 MBq; 38 GBq/mmol) and purified CEF-RSV (37°) GalTase (30 milliunits) in the presence of mM $MnCl_2$ in a total volume of 2.0 mL at 37° for 4 h. At the end of the incubation, the reaction mixture was placed on ice and dialyzed against water (0–4°) to remove intact nucleotide precursors prior to lyophilization.

Determination of glycopeptide heterogeneity and isolation of [^{3}H]galactosylated glycopeptides. — Glycopeptides were prepared from intact glycoproteins after galactosylation by exhaustive protease digestion in a manner analogous to that of Tai *et al.*[29] to ensure complete removal of peptide cores. Thus, glycopeptides (10 mg/mL) were subjected to three cycles of digestion with 1% (w/v) Pronase (Sigma) at 45° for 48 h with interval chromatography on a column (2.6 × 100 cm) of Bio-Gel P-30, equilibrated and developed in water (15 mL/h; 15-min fractions), and monitoring of neutral hexose by the phenol–H_2SO_4 method[30] and radioactivity (D-[^{3}H]galactose and L-[^{14}C]leucine). Removal of peptide was complete after the third

cycle of Pronase digestion, as indicated by the absence of [^{14}C]leucine comigrating with [^{3}H]galactose.

In initial studies, glycopeptides were chromatographed, after final Pronase digestion, on Dowex 50-X8 to separate neutral and acidic oligosaccharides prior to Bio-Gel P-30 chromatography. However, this step was not found to increase resolution beyond that achieved by direct Bio-Gel P-30 chromatography of the complete digestion mixture. Accordingly, after the final Pronase digestion, glycopeptides were chromatographed on a column (150 cm × 0.9 cm) of Bio-Gel P-30, developed in distilled water, and 10mM ammonium acetate or 0.1M pyridine acetate, pH 5.0 (10 mL/h; 10-min fractions), with monitoring as described earlier. Fractions corresponding to individual peaks were lyophilized and chromatographed on a column (0.9 × 100 cm) of Bio-Gel P-6 or P-4, developed as just described to give fractions that were lyophilized prior to compositional and partial structural analysis. The homogeneity of neutral oligosaccharides was assessed by l.c., with a Varian 5000 HPLC instrument equipped with columns of MicroPak AX-10 (Varian Associates, Inc., Palo Alto, CA.) under the conditions previously described[31]. The columns were standardized with neutral high-mannose oligosaccharides prepared from ovalbumin by techniques previously described[26].

RESULTS

GalTase was purified from RSV-transformed fibroblasts. Initial purification was accomplished by sequential chromatography on α-lactalbumin–Sepharose 4B and *N*-acetylglucosamine–Sepharose 4B resins, as previously described[27], to achieve a preparation enriched 32 000-fold with an overall yield of 72%. Final purification was achieved by chromatography on an affinity resin utilizing an anti-GalTase monoclonal antibody (V10) coupled to Sepharose 4B to yield a total 132 000-fold purification. The results of the CEF-RSV (37°) purification are summarized in Table I.

Purified CEF-RSV (37°) GalTase enzyme was found to migrate as a diffuse band with a mean M_r 52 000 on sodium dodecyl sulfate–PAGE gels. Kinetic

TABLE I

PURIFICATION OF UDP-D-GALACTOSE: 2-ACETAMIDO-2-DEOXY-β-D-GLUCOPYRANOSYL 4-β-D-GALACTOSYL-TRANSFERASE FROM CEF-RSV (37°)

Fraction	*Specific activity* ($pmol \cdot mg^{-1} \cdot h^{-1}$)	*Yield* (%)	*Purification* (*fold*)
Soluble sonicate	1.3×10^4	100	1
α-Lactalbumin–Sepharose	2.5×10^8	103	1920
GlcNAc–Sepharose	6.3×10^9	72	32 800
V10–Sepharose[a]	1.7×10^{10}	73	132 000

[a]Anti-GalTase monoclonal antibody coupled[28] to Sepharose 4B.

parameters were defined and included a relatively broad pH optimum (pH 7.0–8.2) and absolute requirement for Mn^{2+}, and K_m UDP-D-galactose 1.1μM and asialogalactofetuin 200μM at 37° by Lineweaver–Burk determination.

Cellular glycoproteins were solubilized, after metabolic labelling with [^{14}C]leucine, from nontransformed fibroblasts CEF-RSV (37°), CEF (41°), CEF-RSV (41°), as well as transformed cells CEF-RSV (37°). Following sonication, 79–88% of the total labelled material in the cell pellet was found in the supernatant after centrifugation, indicating effective solubilization. Subsequently, glycoproteins were galactosylated with a radiolabelled nucleotide precursor and GalTase purified from CEF-RSV (37°). These procedures yielded labelled, cellular glycoproteins of sp. act. 1.1, 1.4, 0.7, and 0.9 · 10^7 c.p.m./mg of protein for CEF-RSV (37°), CEF-RSV (41°), CEF (37°), and CEF (41°), respectively. Glycopeptides were then prepared by repeated Pronase digestion, which effectively removed all radiolabelled

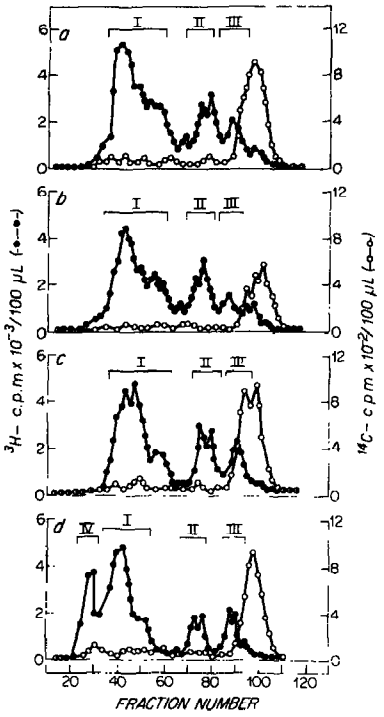


Fig. 1. Bio-Gel P-30 chromatography of cellular glycopeptides. Glycoproteins from 25–50 semiconfluent roller bottles of each cell type, metabolically labelled with [^{14}C]leucine and subsequently galactosylated with [^{3}H]galactose *in vitro*, were digested with Pronase for 3 cycles, and the material remaining after the third cycle was applied to Bio-Gel P-30. Elution was monitored for [^{3}H]galactose (—●—●—) and [^{14}C]leucine (—○—○—) by determination of radioactivity in 100-μL aliquots in Aquasol II (10 mL), as well as for neutral hexose[30] (not shown). Fractions were pooled as indicated; early-, mid-, and late-eluted peaks for each cell type were numbered I, II, and III, respectively. An earlier eluted peak, found only in CEF-RSV (37°), was designated IV: (a) CEF (37°), (b) CEF (41°), (c) CEF-RSV (41°), and (d) CEF-RSV (37°).

amino acid. The galactosylated, cellular glycopeptides were subsequently chromatographed in Bio-Gel P-30 to assess the extent of heterogeneity.

Not surprisingly, all cell types were found to yield varied mixtures of glycopeptides as shown in Fig. 1. However, a small number of glycopeptide species, defined by Bio-Gel P-30 chromatography and designated Glycopeptides I, II, and III, were found to comprise a preponderance of the recovered radiolabelled material, *viz.*, 51–67% of the total glycopeptide in all cells. Comparison of Bio-Gel P-30 chromatographic profiles demonstrated a close similarity between all phenotypically nontransformed CEF, including CEF (37°), CEF (41°), and CEF-RSV (41°).

The three preponderantly labelled peaks (I, II, and III) from each cell type were pooled, as indicated in Fig. 1, and subjected to chromatography on Bio-Gel P-10. This technique resolved each of the major peaks obtained on Bio-Gel P-30 chromatography into two discrete glycopeptides, as depicted in Fig. 2. Peak II was incompletely resolved on Bio-Gel P-10 but could be separated into two discrete components by subsequent chromatography on Bio-Gel P-6 (data not shown).

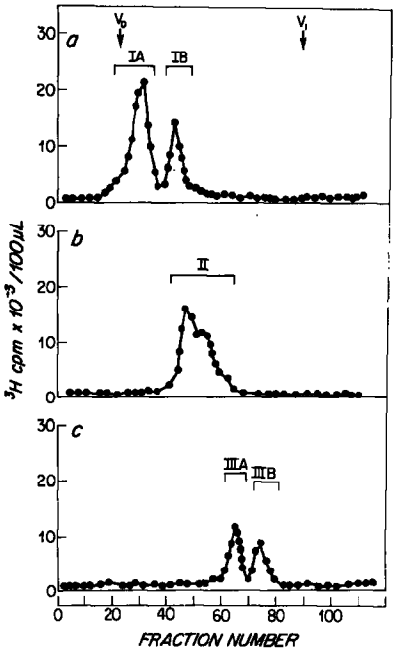


Fig. 2. Bio-Gel P-10 chromatography of CEF (37°) glycopeptides. Cellular glycopeptides I, II, and III, isolated from CEF (37°) by Bio-Gel P-30 chromatography as described in Fig. 1, were lyophilized, resuspended in water (1.0 mL), and individually chromatographed on a column (1.5 × 100 cm) of Bio-Gel P-10, developed with water (5.0 mL/h, 0.5-mL fractions). The elution was monitored for [^{3}H]galactose by determination of radioactivity in 100-µL aliquots in Aquasol II (10 mL), as well as for neutral hexose[30] (not shown). The peaks were pooled and numbered as indicated. The panels represent the chromatography of glycopeptides previously separated on Bio-Gel P-30 (see Fig. 1): (a) Peak I, (b) Peak II, asnd (c) Peak III. V_o, void volume; V_i, included volume.

TABLE II

CONTENT OF CELLULAR GLYCOPEPTIDES IN TRANSFORMED AND NONTRANSFORMED CHICK EMBRYO FIBROBLASTS

Cell	*Temp. (degrees)*	*Glycopeptide[a] (μg/mg of total cellular glycopeptide)*						
		IA	*IB*	*IIA*	*IIB*	*IIIA*	*IIIB*	*IV*
CEF	41	182	92	63	49	35	28	[b]
	37	195	102	68	54	38	30	[b]
CEF-RSV	41	205	110	59	47	45	34	[b]
	37	198	94	62	40	31	72	78

[a]Glycopeptides identified by sequential chromatography on Bio-Gel P-30, P-10, and P-6 or P-4 as indicated in Figs. 1 and 2. [b]Not detectable.

Thus, the total sequential chromatography resolved cellular glycopeptides into six distinct major components, designated glycopeptides IA, IB, IIA, IIB, IIIA, and IIIB. Although there were some small differences in the relative amounts of each of the six glycopeptides among the nontransformed cell types, none appeared to be statistically significant (Table II).

The glycopeptides obtained from transformed CEF-RSV (37°) were studied in a similar manner. The sequential chromatography demonstrated the presence of the same six glycopeptides found in nontransformed cells (IA, IB, IIA, IIB, III, and IIIB) (data not shown). However, the relative amounts of the glycopeptides appeared to differ from those in the nontransformed cells (Table II). Thus, glycopeptide IIIB was significantly enriched relative to glycopeptides IIA and IIIA. However, it should be noted that Bio-Gel P-30 chromatography of CEF-RSV (37°) glycopeptides also disclosed an additional, early eluted peak, designated glycopeptides IV (Fig. 1d), which was not found in significant amounts in nontransformed cells. No comparable glycopeptides, either labelled or unlabelled (as monitored by assay for neutral hexose) was recovered from Bio-Gel P-30 chromatography of glycopeptides from nontransformed cells. This material migrated as a single peak during subsequent chromatography on Bio-Gel P-10 and P-6 resins (data not shown). Nonetheless, the compositions of other glycopeptides from transformed and nontransformed cells were the same when assessed by gas–liquid chromatography of the per(trimethylsilyl) derivatives prepared as previously described[18,19], (*cf.* Tables III and IV).

As shown in Tables III and IV, all glycopeptides demonstrated composition compatible with neutral complex oligosaccharides that were fucosylated to a minor degree. The compositional analysis of these substances suggests that they represented a family of bi- and tri-antennary structures by virtue of the relative molar amounts of galactose, based on the calculated molar weight of the glycopeptides derived from Bio-Gel P-10 chromatography using oligosaccharide standards. The molar ratios of galactose further suggest that galactosylation following the *in vivo*

TABLE III

CARBOHYDRATE COMPOSITION (MOL/MOL)[a] OF CELLULAR GLYCOPEPTIDES FROM CEF (37°)

Sugar	*Glycopeptides*[b]					
	IA	*IB*	*IIA*	*IIB*	*IIIA*	*IIIB*
Fucose	0.3	0.4	0.4	0.2	0.3	0.3
Mannose	2.7	2.8	2.7	3.1	2.6	2.9
Galactose	2.8	3.1	1.9	2.2	1.7	1.9
Glucose	[c]	[c]	[c]	[c]	[c]	[c]
Glucosamine	5.2	4.9	4.2	3.9	4.0	3.8
Galactosamine	[c]	[c]	[c]	[c]	[c]	[c]
Sialic acid	0.2	[c]	<0.1	0.1	[c]	[c]

[a]The carbohydrate composition was determined on the per(trimethylsilyl) derivatives prepared after hydrolysis as described[15]. The molar ratios were calculated from the glycopeptide dry-weight and mol. wt. determined by gel chromatography (SD ±12%). [b]CEF (37°) glycopeptides were isolated and identified by sequential column chromatography as described in text and Figs. 1 and 2. [c]Not detected.

TABLE IV

CARBOHYDRATE COMPOSITION (MOL/MOL)[a] OF CELLULAR GLYCOPEPTIDES FROM CEF-RSV (37°)

Sugar	*Glycopeptides*[b]						
	IA	*IB*	*IIA*	*IIB*	*IIIA*	*IIIB*	*IV*
Fucose	0.3	0.2	0.2	0.3	0.4	0.3	0.2
Mannose	3.2	3.0	3.2	2.7	2.2	2.1	2.9
Galactose	2.9	3.3	2.2	1.8	2.0	1.8	4.1
Glucose	[c]	[c]	[c]	[c]	[c]	[c]	[c]
Glucosamine	4.9	4.7	4.0	3.8	4.3	3.9	6.2
Galactosamine	[c]	[c]	[c]	[c]	[c]	[c]	[c]
Sialic acid	<0.1	0.2	[c]	[c]	[c]	[c]	<0.1

[a]The carbohydrate composition was determined on the per(trimethylsilyl) derivatives prepared after hydrolysis as described[15] (SD ±12%). [b]CEF-RSV (37°) glycopeptides were isolated and identified by sequential column chromatography as described in text and Figs. 1 and 2. [c]Not detected.

incubation with GalTase is complete under the conditions of nucleotide sugar donor excess and prolonged incubation employed, *i.e.*, all branches were fully galactosylated. From the present experiments, it cannot be inferred to what extent the bi- and tri-antennary compounds were already partially or completely galactosylated prior to *in vitro* glycosylation. It should be noted that treatment of the six glycopeptides with diplococcal exo-β-D-galactosidase resulted in the removal of all detectable galactose, which supports the conclusion that each radiolabeled residue was added to a discrete oligosaccharide branch and effectively precludes the presence of extended branches containing within them more than one galactose residue.

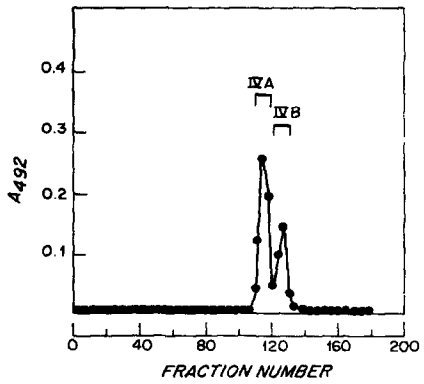


Fig. 3. Bio-Gel P-4 chromatography of CEF-RSV (37°) glycopeptide IV after β-D-galactosidase digestion. CEF-RSV (37°) glycopeptide IV, isolated by sequential chromatography on Bio-Gel P-30, P-10, and P-6 (Fig. 1d), was digested with exo-β-D-galactosidase, as described in the text, and chromatographed on a column of Bio-Gel P-4, as described in the legend to Fig. 2. The elution was monitored for neutral hexose by the phenol–H_2SO_4 method[30]. The fractions were pooled as indicated and designated glycopeptides IVA and IVB.

More detailed structures of the high-molecular-weight glycopeptides (IV) isolated from CEF-RSV (37°) were partially elucidated by a combination of sequential exoglycosidase digestion and permethylation analysis (see Table IV). Digestion of glycopeptide IV with exo-β-D-galactosidase effectively removed all labelled galactosyl groups, although some apparently unlabelled galactose residues (<0.1 mol/mol of initial glycopeptide) remained as determined by g.l.c. compositional analysis. The mean amount of galactose removed by the exoglycosidases was ~3.5 mol of galactose/mol. These observations suggested the presence of a heterogeneous population of oligosaccharides, perhaps encompassing extended chains with intra-chain galactose residues. This suggestion is supported by the finding of tri-*O*-methylgalactose in the methylation analysis. Chromatography of glycopeptide IV

TABLE V

METHYLATION ANALYSIS OF OLIGOSACCHARIDE IV ISOLATED FROM CEF-RSV (37°) CELLS

Methylated sugar[a]		*Molar ratio*[b]
Fucitol,	2,3,4-tri-*O*-methyl-	0.3
Galactitol,	2,3,4,5-tetra-*O*-methyl-	3.8
	2,4,6-tri-*O*-methyl-	0.3
Mannitol,	3,4,6-tri-*O*-methyl-	0.6
	3,6-di-*O*-methyl-	0.4
	3,4-di-*O*-methyl-	0.8
	2,4-di-*O*-methyl-	1.0
2-Deoxy-2-(*N*-methyl-	3,5-di-*O*-methyl-	4.2
acetamido)-glucitol	1,3,4-tri-*O*-methyl-	0.5

[a]Analyzed as alditol acetates. [b]Expressed relative to 2,4-di-*O*-methylmannitol.

TABLE VI

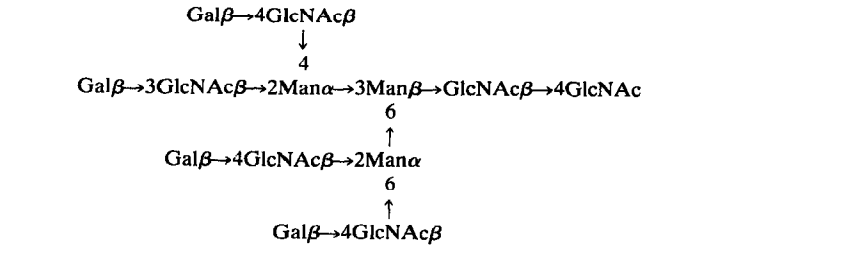
SUGAR COMPOSITION AND GLYCOSIDASE DIGESTION OF CEF-RSV (37°) GLYCOPEPTIDES IV, IVA, AND IVB

Glycopeptide	*Glycosidase treatment*	*Sugar composition of residual glycopeptide*[a]			
		Fucose	*Galactose*	*GlcNAc*	*Mannose*
IV[b]	Intact	0.2	4.1	6.2	3.0
	α-D-Galactosidase	0.3	3.8	6.0	3.0
	β-D-Galactosidase	0.2	0.4	5.8	3.0
IVA[c]	"Intact"	0.2	<0.1	5.9	3.0
	N-Acetyl-β-D-hexosaminidase	0.3	<0.1	1.7	3.0
	α-D-Mannosidase	0.4	<0.1	6.1	3.0
	β-D-Mannosidase	0.3	<0.1	5.7	3.0
	N-Acetyl-β-D-hexosaminidase, then α-D-mannosidase,	0.2	<0.1	1.8	1.1
	and then β-D-mannosidase	0.3	<0.1	2.1	<0.2
IVB[c]	"Intact"	0.3	1.1	6.2	3.0
	N-Acetyl-β-D-hexosaminidase	0.4	0.9	3.1	3.0
	N-Acetyl-β-D-hexosaminidase, then β-D-galactosidase,	0.2	<0.1	3.2	3.0
	then *N*-acetyl-β-D-hexosaminidase,	0.3	<0.1	2.3	3.0
	and then α-D-mannosidase	0.2	<0.1	1.8	0.9

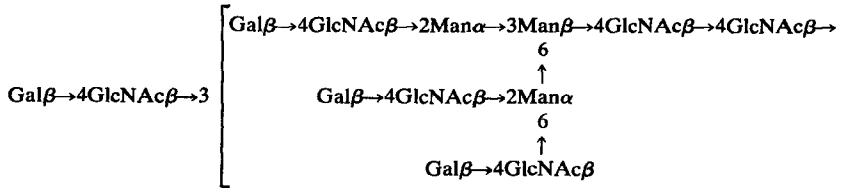
[a]Composition determined by g.l.c. as described; molar ratio calcultaed from initial calculated mol. wt. as described in the text, presuming 3 mannose residues/mol glycopeptide. [b]Glycopeptide IV recovered from Bio-Gel P-30 chromatography of glycopeptides prepared from CEF-RSV (37°). [c]Glycopeptides IVA and IVB, separated and recovered from Bio-Gel P-4 column chromatography after treatment of glycopeptide IV with exo-β-D-galactosidase; thus, "intact" IVA and IVB are products of prior β-D-galactosidase digestion.

on Bio-Gel P-4 following digestion with β-D-galactosidase demonstrated the presence of two glycopeptide peaks (A and B) that could have been formed by removal of 3 and 4 galactose residues, respectively, from the parent glycopeptide (Fig. 3 and Table VI). The molar ratio of the two peaks was ~3:1. Digestion of glycopeptide IVB with *N*-acetyl-β-D-hexosaminidase resulted in removal of four residues of *N*-acetylglucosamine with subsequent apparent exposure of two α- and one β-D-linked mannosyl residues, as determined by further glycosidase digestion (Table VI). These data, in conjunction with the methylation studies carried out on the parent glycopeptide mixture, are compatible with the tetraantennary complex structure **1**. Glycopeptide IVA was found to contain a single (unlabelled) galactose residue after treatment with exo-β-D-galactosidase. Treatment of the product with *N*-acetyl-β-D-hexosaminidase resulted in exposure of the remaining galactose residue to the action of exo-β-D-galactosidase. In conjunction with the methylated sugars found in the initial glycopeptide IV, these data are consistent with a family of triantennary complex oligosaccharides comprised of lactosaminyl groups linked at O-3′. The present data are inadequate to determine the relative amount of substitution of the different branches or between two core,

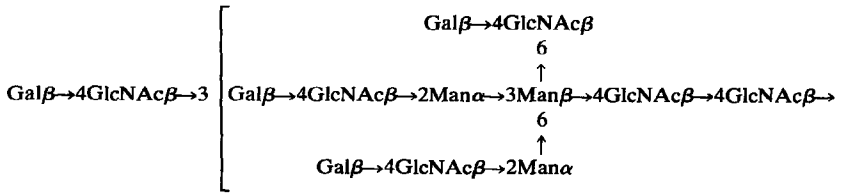
biantennary structures; thus the glycopeptide IVB could represent a mixture of unknown relative proportions of **2** and **3**.



1*



2



3

DISCUSSION

Past investigations to define malignant transformation related changes in glycosyltransferase activities or glycopeptides have yielded widely disparate results. In part, many previous experimental approaches have been limited to the examination of either enzymic activities or isolated substrate–products. In the studies reported herein, GalTase activity from RSV-transformed, primary fibroblast cultures was isolated in conjunction with the identification of relevant endogenous substrates. Sequential affinity chromatography of GalTase activity, solubilized from transformed fibroblasts, yielded a preparation having kinetic and physical characteristic properties closely resembling those of other soluble, tissue- and cell-derived

*In abbreviated structures, the D configuration, pyranose form, and linkage at C-1 of Gal, GlcNAc, and Man are assumed.

UDP-D-galactose: 2-acetamido-2-deoxy-β-D-glucopyranosyl 4-β-D-galactosyltransferases[12,26,27]. Although multiple discrete bands were not observed when the purified preparation was examined by sodium dodecyl sulfate–PAGE electrophoresis, these observations do not preclude the possibility of multiple GalTase activities which would be copurified by the procedures used. The issue is of particular interest in view of the apparent range of endogenous substrates present in the RSV-transformed cell (as well as nontransformed counterparts). Although the presence of multiple GalTase activities cannot be excluded, the ability of the purified GalTase preparation to transfer a β-D-galactosyl group to various glycopeptides, ranging from bi- to tetra-antennary structures, suggest alternatively that the purified GalTase is indeed relatively broad in its substrate specificity. More detailed kinetic studies utilizing individual glycopeptide substrates should provide insight into the mechanism of the production of the range of galactosylated glycopeptides. It will also be of particular interest to determine whether there is preferential galactosyl addition to the various branches by use of conditions limiting galactosylation[32].

Not surprisingly, both RSV-transformed CEF and their nontransformed counterparts encompassed a range of complex, neutral glycopeptide structures as potential GalTase acceptor substrates[1–5,7–9]. It is noteworthy that the transformed cells contained a range of more highly branched, or extended structures, or both, which were not isolated from uninfected fibroblasts or even infected cells when they were maintained in nonpermissive growth conditions. The tetraantennary and extended triantennary glycoproteins recovered from CEF-RSV (37°) resemble those found to be enriched in other virally transformed, primary cultures and cell lines[33]. The possibility that these structures were present in a fully galactosylated form in nontransformed cells is unlikely in view of the failure to recover unlabelled glycopeptides having similar elution properties from these cell types after Bio-Gel P-30 chromatography. It should be noted that the quantitative limits of methods employed in these studies do not preclude the presence of these structures in the nontransformed cells, but suggest that, if present, their concentration is at least five-fold less than that in the transformed fibroblast. Similarly, these methods do not exclude the presence of additional minor transformation- or nontransformation-related, endogenous substrates of the GalTase activity. Insofar as galactose is present as nonreducing group, the presence of transformation-associated, highly branched structures may indicate distinctive alterations in glycosyltransferase activities involved in the addition of more proximal residues found at sites of branching. The presence of more highly branched chains could reflect the presence of "unique" glycosyltransferases (*e.g.*, *N*-acetylglucosaminyltransferases) or alteration in the relative amounts of normal enzymic entities.

It should be noted that glycopeptide structures isolated from transformed and nontransformed cells were equally effective as acceptor substrates for the CEF-RSV (37°) GalTase. Similar studies using GalTase purified from noninfected CEF yielded comparable results, *i.e.*, equivalent galactosylation of transformed and non-

transformed cell-derived substrates. The rates of galactosylation by GalTase isolated from the nontransformed CEF were also comparable to those observed with the CEF-RSV (37°) GalTase[34]. Collectively, these observations suggest that any alterations in the extent of galactosylation of glycoprotein following transformation may primarily reflect differences in the amount and, perhaps, the range of acceptor substrates. However, further work is needed to define the extent of transformed-cell GalTase heterogeneity and, within this context, acceptor specificity. At the same time, these studies underscore the importance of examining both glycosyltransferases and acceptor substrates to define the basis of changes in cellular glycoproteins in relation to alterations in growth.

ACKNOWLEDGMENT

The authors acknowledge the superb technical assistance of Ms. Kathy Lynch in the performance of these studies.

REFERENCES

1 C. A. BUCK, M. C. GLICK, AND L. WARREN, *Biochemistry*, 9 (1970) 4567–4576.
2 C. A. BUCK, M. C. GLICK, AND L. WARREN, *Biochemistry*, 10 (1971) 2175–2180.
3 M. M. C. LAI AND P. H. DUESBERG, *Virology*, 50 (1972) 359–372.
4 C. RIEBER AND J. C. IRWIN, *Cancer Res.*, 34 (1974) 3469–3473.
5 S. G. LEONARD, A. H. HALE, D. E. ROLL, H. E. CONRAD, AND M. J. WEBER, *Cancer Res.*, 38 (1978) 185–188.
6 B. S. LITIN AND W. J. GRIMES, *Cancer Res.*, 43 (1983) 2131–2137.
7 G. COSSU, L. WARREN, D. BOETTIGER, H. HOLTZER, AND M. PACIFICI, *J. Biol. Chem.*, 257 (1982) 4463–4468.
8 L. A. HUNT, W. LAMPH, AND S. E. WRIGHT, *J. Virol.*, 37 (1981) 207–215.
9 L. A. HUNT, S. E. WRIGHT, J. R. ETCHISON, AND D. F. SUMMERS, *J. Virol.*, 29 (1979) 336–343.
10 M. C. GLICK, *Biochemistry*, 18 (1979) 2525–2532.
11 J. HAKIMI AND P. H. ATKINSON, *Biochemistry*, 19 (1980) 5619–5624.
12 T. FEIZI AND R. A. CHILDS, *Trends Biochim. Sci.*, (1985) 24–28.
13 M. M. WEISER, W. A. KLOHS, D. K. PODOLSKY, AND J. R. WILSON, in M. I. HOROWITZ (Ed.), *The Glycoconjugates*, Vol. VIB, Academic Press, New York, 1983, pp. 301–335.
14 M. BERTOLINI AND W. PIGMAN, *J. Biol. Chem.*, 242 (1967) 3776–3781.
15 D. K. PODOLSKY AND M. M. WEISER, *Biochem. J.*, 146 (1978) 213–221.
16 S. I. HAKOMORI, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
17 A. SLOMIANY AND B. L. SLOMIANY, *J. Biol. Chem.*, 253 (1978) 7307–7306.
18 K. STELLNER, H. SAITO, AND S. HAKOMORI, *Arch. Biochem. Biophys.*, 155 (1973) 464–472.
19 B. L. SLOMIANY AND A. SLOMIANY, *Eur. J. Biochem.*, 90 (1978) 39–49.
20 B. L. SLOMIANY, V. L. N. MURTY, AND A. SLOMIANY, *J. Biol. Chem.*, 255 (1980) 9719–9723.
21 E. V. CHANDRASEKARAN, M. DAVILA, D. W. NIXON, M. GOLDFARB, AND J. MENDICINO, *J. Biol. Chem.*, 258 (1983) 7213–7222.
22 D. K. PODOLSKY, *J. Biol. Chem.*, 260 (1985) 8262–8271.
23 L. R. GLASGOW, J. C. PAULSON, AND R. L. HILL, *J. Biol. Chem.*, 252 (1977) 8615–8623.
24 K.-I. INUI, L. G. TILLOTSON, AND K. J. ISSELBACHER, *Biochim. Biophys. Acta*, 598 (1980) 616–627.
25 D. K. PODOLSKY AND M. M. WEISER, *Biochem. Biophys. Res. Commun.*, 65 (1975) 545–551.
26 D. K. PODOLSKY, M. M. WEISER, J. C. WESTWOOD, AND M. GAMMON, *J. Biol. Chem.*, 252 (1977) 1807–1814.
27 D. K. PODOLSKY AND M. M. WEISER, *J. Biol. Chem.*, 254 (1979) 3983–3990.
28 D. K. PODOLSKY AND K. J. ISSELBACHER, *Proc. Natl. Acad. Sci. U.S.A.*, 81 (1984) 2529–2533.

29 T. TAI, K. YAMASHITA, M. OGATA-ARAKAWA, N. KOIDE, T. MURAMATSU, S. SWASHITA, Y. INOUE, AND A. KOBATA, *J. Biol. Chem.*, 25 (1975) 8569–8575.
30 J. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350–356.
31 S. J. MELLIS AND J. BAENZIGER, *Anal. Biochem.*, 114 (1981) 276–280.
32 W. M. BLANKEN, A. VAN VLIET, AND D. H. VAN DEN EIJNDEN, *J. Biol. Chem.*, 259 (1984) 15 131–15 135.
33 K. YAMASHITA, T. OHKURA, Y. TACHABARI, S. TAKASAKI, AND A. KOBATA, *J. Biol. Chem.*, 259 (1984) 10 834–10 840.
34 D. K. PODOLSKY, unpublished results.